PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

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PCT

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

Date of mailing (*day/month/year*) 06 APR 2006		
Applicant's or agent's file reference 62310WO (71699)	**FOR FURTHER ACTION** See paragraph 2 below	
International application No. PCT/US05/09391	International filing date (*day/month/year*) 22 March 2005 (22.03.2005)	Priority date (*day/month/year*) 22 March 2004 (22.03.2004)
International Patent Classification (IPC) or both national classification and IPC IPC(8): C12Q 1/68; C12P 19/34 and US Cl.: 435/6, 91.2		
Applicant THE JOHNS HOPKINS UNIVERSITY		

1. This opinion contains indications relating to the following items:

- ☒ Box No. I — Basis of the opinion
- ☐ Box No. II — Priority
- ☐ Box No. III — Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☒ Box No. IV — Lack of unity of invention
- ☒ Box No. V — Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI — Certain documents cited
- ☒ Box No. VII — Certain defects in the international application
- ☒ Box No. VIII — Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1*bis(b)* that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

3. For further details, see notes to Form PCT/ISA/220.

Name and mailing address of the ISA/ US	Date of completion of this opinion	Authorized officer
Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (571) 273-3201	19 January 2006 (19.01.2006)	Stephen Kapushoc Telephone No. 571-272-1600

Form PCT/ISA/237 (cover sheet) (April 2005)

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Box No. I Basis of this opinion

1. With regard to the **language**, this opinion has been established on the basis of:
   - ☒ the international application in the language in which it was filed
   - ☐ a translation of the international application into _____, which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

2. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application and necessary to the claimed invention, this opinion has been established on the basis of:

   a. type of material
      - ☐ a sequence listing
      - ☐ table(s) related to the sequence listing

   b. format of material
      - ☐ on paper
      - ☐ in electronic form

   c. time of filing/furnishing
      - ☐ contained in the international application as filed.
      - ☐ filed together with the international application in electronic form.
      - ☐ furnished subsequently to this Authority for the purposes of search.

3. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table(s) relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

4. Additional comments:

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Box No. IV Lack of unity of invention

1. ☒ In response to the invitation (Form PCT/ISA/206) to pay additional fees the applicant has, within the applicable time limit:
   - ☐ paid additional fees
   - ☐ paid additional fees under protest and, where applicable, the protest fee
   - ☐ paid additional fees under protest but the applicable protest fee was not paid
   - ☒ not paid additional fees
2. ☐ This Authority found that the requirement of unity of invention is not complied with and chose not to invite the applicant to pay additional fees.
3. This Authority considers that the requirement of unity of invention in accordance with Rule 13.1, 13.2 and 13.3 is
   - ☐ complied with
   - ☒ not complied with for the following reasons:

   See the lack of unity section of the International Search Report(Form PCT/ISA/210)

4. Consequently, this opinion has been established in respect of the following parts of the international application:
   - ☐ all parts.
   - ☒ the parts relating to claims Nos. 1-5, 28-74 (species regarding analysis of HIV variant)

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Box No. V Reasoned statement under Rule 43 *bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	Please See Continuation Sheet	YES
	Claims	Please See Continuation Sheet	NO
Inventive step (IS)	Claims	Please See Continuation Sheet	YES
	Claims	Please See Continuation Sheet	NO
Industrial applicability (IA)	Claims	Please See Continuation Sheet	YES
	Claims	Please See Continuation Sheet	NO

2. Citations and explanations:

Please See Continuation Sheet

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Box No. VII Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

Claim 40 is objected to under PCT Rule 66.2(a)(iii) as containing the following defect(s) in the form or contents thereof: in the tenth line of the claim the term 'nucelotide difference' is redundant with 'nucleic acid difference'.

Claim 70 is objected to under PCT Rule 66.2(a)(iii) as containing the following defect(s) in the form or contents thereof: in the second line of the claim the word 'oligonucleotide' is misspelled as 'oliginuclietides'.

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Box No. VIII Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the questions whether the claims are fully supported by the description, are made:

Claim 42 is objected to under PCT Rule 66.2(a)(v) as lacking clarity under PCT Article 6 because claim 42 is indefinite for the following reason(s): The claim is dependent upon claim 41. Claim 41 is a multiple dependent claim that is dependent upon any of claims 1, 6, 14, 23, 28, or 40. However, the subject matter of claim 42 (a nucleotide that ligates to one or more of P1 or P2) is only relevant to the method of claim 40.

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Supplemental Box
In case the space in any of the preceding boxes is not sufficient.

V.1. Reasoned Statements:
The opinion as to Novelty was positive (Yes)with respect to claims 5, 28, 29, 40, 42, 47, 48, 53, 62, 67, 70
The opinion as to Novelty was negative (No) with respect to claims 1-4, 30-39, 41, 43-46, 49-52, 54-61, 63-66, 68,69, 71-74
The opinion as to Inventive Step was positive (Yes)with respect to claims NONE
The opinion as to Inventive Step was negative(NO) with respect to claims 1-5, 28-74
The opinion as to Industrial Applicability was positive (YES) with respect to claims 1-5, 28-74
The opinion as to Industrial Applicability was negative(NO) with respect to claims NONE

V. 2. Citations and Explanations:
Please See Continuation Sheet

Claims 1, 30-39, 41, 43-45, 49-50, 52, 56-58, 60, 63-66, 68, 69, and 71-74 lack novelty under PCT Article 33(2) as being anticipated by Schouten et al (2002).

Schouten et al teaches a ligation/amplification based method (termed multiplex ligation-dependent probe amplification (MLPA)) to analyze nucleic acid sequences, including the characterization of chromosomal aberrations and SNP/mutation detection, that is comprised of all of the method steps of the claimed invention.

Regarding claim 1, the method of Schouten et al (summarized in Fig.2 on p.4) utilizes a primer pair in which each of the primers has a gene specific region (termed 'hybridisation sequence' in the reference) and a primer region (termed 'PCR primer sequence Y' and 'PCR primer sequence X' in the reference). The reference indicates that mutation detection can be accomplished by using a nucleotide difference (as compared to the target nucleic caid sequence) in the gene specific region of one primer (p.11, right col., lns.5-10). The method steps of the reference indicate that the primers of the pair are suitable for ligation to one another (p.2, right col., l.1).

Regarding claims 30-32, Schouten et al teaches that the primer nucleotides responsible for detecting sequence mutations in the target gene are at the 3' end of the primer (p.11, right col., lns.5-7; p.12, left col., l.1).

Regarding claims 33-34, Schouten et al teaches an example of the detection of the CFTR ΔF508 3nt deletion (Fig.8; p.11 - SNP and mutation detection). The portion of the primer that detects the 3nt present in the allele without the deletion satisfies the limitation of the claims which require sizes of between about 1 and about 5 nucleotides in length, and between about 5 and about 30 nucleotides in length.

Regarding claim 35, Schouten et al indicates that probe signal is absent if one probe has a mismatch at the 3' nucleotide (p.11, right col., lns.5-7; p.12, left col., lns.1-3), thus indicating that the supplied primers ligate only if the anticipated sequence is present in the target nucleic acid.

Regarding claims 36-39, Schouten et al teaches the multiplexed use of up to 40 different primer pairs (p.4, right col., l.8; Fig.3) that analyze different portions of different genes (p.11, right col., ln.38).

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Regarding claims 41 and 43, Schouten et al specifically teaches the analysis of the CFTR ΔF508 mutation, which is a deletion mutation.

Regarding claims 44 and 45, Schouten et al teaches that the length of the primers used in the method are typically between 80 and 440 nt (p.3, left col., ln.5), and that the gene specific portion ('hybridisation sequence') of the primers is between 21-30 nt (p.2 left col., ln.25) or 25-43 nt (p.2, right col., last two lines).

Regarding claims 49, 50, and 52, Schouten et al teaches that one of the primers of each primer pair contains a 'stuffer sequence' (Fig.2; p.4, left col., lns.6-9) that is a unique sequence derived from the M13 vector. This stuffer sequence (which is used in the reference as a detection region based on the unique length of each 'stuffer sequence') is also a probe binding region because it is a nucleic acid sequence, composed of a known sequence (because it is derived from the known sequence of the M13 vector), and is unique to the M13 vector and not related to the target nucleic acid.

Regarding claims 56 and 60, Schouten et al teaches the analysis of human chromosomal DNA which is genomic DNA (p.5, right col., lns.15-19).

Regarding claims 57, 58 and 66, Schouten et al teaches that the amplification of ligated primers is by PCR (p.2, right col., lns.7-15), and further teaches that the ligated probes are linearly amplified during the PCR (p.4 - Reaction conditions) thus making the reaction quantitative. The amplification of the ligated primers by PCR would necessarily include bottom strand synthesis (as required by claim 66)

Regarding claims 63,64, and 65, Schouten et al teaches the analysis of the amplification product by slab gel and capillary electrophoresis (p.2, right col., lns.17-20) and specifically mentions the use of ethidium bromide (p.2, right col., ln.22)

Regarding claims 68 and 69, Schouten et al teaches the analysis of the CTFR gene in a DNA sample that is heterozygous for the ΔF508 mutation (Fig.8). Because the sample is heterozygous, the ratio of ΔF508 mutation to wild type sequence in the sample is 1:1. Schouten et al also provides a probe that is detecting the wild-type allele by providing a primer that overlaps the 3 nucleotides present in the wild type gene.

Regarding claims 71-73, Schouten et al teaches the analysis of human nucleic acid samples including sample of DNA from blood (Fig.6).

Regarding claim 74, Schouten et al teaches the identification of the ΔF508 CFTR mutation (Fig.8), thus the method can be used to diagnose the disease cystic fibrosis.

Claims 1-4, 30-39, 43-46, 49-52, 54-57, 59-61, 63-66, 68, and 71-74 lack novelty under PCT Article 33(2) as being anticipated by Belgrader et al WO 97/45559 (1997).

Regarding claims 1-4, Belgrader et al teaches methods to analyze nucleic acid sequences that is comprised of all of the method steps of the claimed invetion. As detailed in Fig. 10, the method comprises the steps of contacting a target nucleic acid with a pair of primers, a portion of each primer is capable of hybridizing to the target nucleic acid, and a portion of each primer serves as a hybridization site for an amplification primer. The two primers can be ligated, and the ligated product is amplified. Belgrader et al teaches that the method is suitable for detecting infectious agents, including viral and microbial agents; the reference specifically mentions HIV (p.61). The reference also indicates that the method is useful for determining the resistance of various infectious agents to drugs (p.60).

Regarding claims 30-32, Belgrader et al teaches that the mutation-specific nucleotides of the primers are a the 3'-end of the first primer, and at the 5'-end of the second primer of the pair (p.72; Fig.24).

Regarding claims 33 and 34, Belgrader et al teaches an example in which the mutation specific portion of the primers is 2 nt, which satisfies the limitation of the claims which require sizes of between about 1 and about 5 nucleotides in length, and between about 5 and about 30 nucleotides in length.

Regarding claim 35, Belgrader et al teaches that ligation of the primers is dependent upon the sequence of the target nucleic acid (Fig. 12).

Regarding claims 36- 39, Belgrader et al teaches that the method can be multiplexed to contain multiple primer pairs to analyze multiple sequences (Fig.10; p.85 - Example 9).

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Regarding claim 43, Belgrader et al teaches that the method is useful for the detection of single nucleotide polymorphisms (p.86, Table 10).

Regarding claims 44 and 45, Belgrader et al teaches the characteristics of the primers used for the method, indicating that the total length can be about 40 nucleotides in length, and that the gene specific portion is about 24 nucleotides in length (Fig.24; pp.71-72).

Regarding claim 46, Belgrader et al specifically teaches the use of DNA zipcode primer binding sites for the amplification of ligated primers (Fig.12; p.90).

Regarding claims 49-52, Belgrader et al teaches a method to analyze the amplified ligation products via capture on an addressable array (Fig.13, p.63-65). The disclosure of Belgrader et al discusses that the amplified ligation products have an addressable array-specific portion, and that the amplified products can be bound to a complementary probe that is bound to a solid support.

Regarding claims 54-55, Belgrader et al teaches that the primers used for the detection of target nucleic acids can be labeled with a florescent moiety (p.83-84; Fig.4)

Regarding claims 56-57, 59, and 60, Belgrader et al teaches the analysis of a provided genomic DNA sample(p.66 - Example 1), and also teaches that the amplification of ligated primers is by PCR (p.75; Fig. 10). Belgrader also teaches the removal of non-ligated primers (which would include primers not bound to target nucleic acid) via an exonucelase digestion (Fig. 12).

Regarding claim 61, Belgrader et al teaches the amplification of the target nucleic acid sample via PCR prior to analysis by the primer ligation method (p.82; Fig.4).

Regarding claims 63-65, Belgrader et al teaches several methods to analyze the reaction product of the probe Ligation reaction, specifically mentioning capillary electrophoresis (Fig.1), and also gel electrophoresis and staiing with ehtidium bromide (p.75).

Regarding claim 66, Belgrader et al teaches amplifcation of ligated probes via PCR, which would involve the systhesis of the bottom strand of the PCR product.

Regarding claim 68, Belgrader et al teaches the anaylsis of DNA samples that are heterozygous for a particular allele, which would have a 1:1 ratio of mutated to wild type nucleic acid (p.85 - Example 9; Fig.18).

Regarding claims 71-74, Belgrader et al teaches the analysis of nucleic acid from humans that is derived from blood (p.66), and also indicates that the method is operable to detect infectious disease minor variants (p.61).

Claim 5 lacks an inventive step under PCT Article 33(3) as being obvious over Schouten et al (2002) in view of Beck et al (2003). Schouten et al teaches a method for detecting specific nucleic acid sequences using pairs of primers the ligation of which are dependent upon the sequence of a target nucleic acid. Schouten does not specifically teach the analysis of the K103N drug resistant HIV viral variant (claim 5).

Beck et al teaches an oligonucleotide ligation assay (Fig.1, p.5) for the analysis of HIV drug resistant mutation including K103N (p.11, Table 3).

It would have been obvious to have used the method of Schouten et al to analyze the same nucleic acid mutations in HIV discussed in Beck et al. One would have been motivated to combine these methods to provide alternative methods for the analysis of the HIV K103N mutation. One would have had a reasonable expectation of success because Beck shows successful analysis using a similar primer ligation based assay.

Claims 28, 29, and 47 lack an inventive step under PCT Article 33(3) as being obvious over Belgrader et al 1997) in view of Kapala et al (2000). Belgrader et al teaches a method for detecting nucleic acid variants using pairs of primers that are ligatable depending on the sequence present in a target nucleic acid sample. Belgrader et al does not teach the use of pooled samples (claims 28 and 29), or an analysis using ligase chain reaction (claim 47).

Kapala et al teaches the pooling of sample for the analysis of cervical swabs for the detection of C. trachomatis using ligase chain reaction.

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It would have been obvious to have modified the methods of Belgrader et al to have used the sample pooling techniques and the ligase chain reaction methods of Kapala et al. One would have been motivated to do so because Kapala et al teaches that sample pooling is cost effective (p.2482, Table 4). One would have had a reasonable expectation of success because Kapala et al shows that ligase chain reaction can accurately detect the infectious agent (p2480, Tale 1).

Claims 40 and 42 lack an inventive step under PCT Article 33(3) as being obvious over Schouten et al (2002) in view of US Patent 5,185,243 to Ullman et al (1993). Schouten et al teaches a method for detecting nucleic acid variants using pairs of primers that are ligatable depending on the sequence present in a target nucleic acid sample. Schouten et al does not teach the use of primers that are separated by a gap and providing the reaction mixture with a nucleotide to fill in the gap.

Ullman et al teaches method for detection of specific nucleic acid sequences. Ullman et al teaches the use of primers that bind to non-contiguous regions of a target nucleic acid, and are subject to a chain extension reaction by providing the appropriate nucleotides to fill in the gap between the non-contiguous primers (c.9 l.59 - c.10 l5; c.28 lns.4-41).

It would have been obvious to have modified the method of Schouten et al to have used the non-contiguous primers and fill in reaction of Ullman et al. One would have bee motivated to do so because Ullman asserts that the method is useful for detecting specific nucleic acids. One would have had a reasonable expectation of success because Ullman provides examples of the use of the technique for the analysis of nucleic acids (col.23 - col.27).

Claim 48 lacks an inventive step under PCT Article 33(3) as being obvious over Belgrader et al (1997) in view of Whitcombe et al (1999). Belgrader et al teaches the detection of specific nucleic acids using ligatable primers that are amplified and detected via capillary electrophoresis or hybridization to a probe array. Belgrader et al does not teach the analysis of ligation product formation using scorpion probes.

Whitcombe et al teaches the detection of PCR product using an allele specific hybridization primer called a Scopion primer (p804, left column, last paragraph; Fig.1).

It would have been obvious to have modified the method of Belgrader et al to have used the Scorpion primers of Whitcombe et al to detect the amplified ligation product. One would have been motivated to do so in order to provide an alternative method for detecting primers that have ligated dependent upon the target nucleic acid sequence. One would have had a reasonable expectation of success because Whitcombe provides examples of the use of Scorpion primers for the detection of amplicons (Fig.2).

Claims 53 and 62 lacks an inventive step under PCT Article 33(3) as being obvious over Belgrader et al (1997) in view of Favis et al (2000). Belgrader et al teaches the detection of specific nucleic acids using ligatable primers that are amplified and via hybridization to a probe array. Belgrader et al does not teach the analysis of ligation products using a detection region comprised of a DNA zipcode.

Favis et al teaches the detection of target nucleic acids using pairs of primers which are ligated then amplified and detected using a zip-cod based microarray system (p562, left column, lns.22-25). The probes on the array hybridize to the zip-code detection region portion of the ligase detection reaction (LDR) primers (p.562, left col., lns33-39).

It would have been obvious to have modified the method of Belgrader et al to have used the DNA zipcode detection method of Favis et al to detect the amplified ligation product. One would have been motivated to do so in order to provide an alternative method for detecting ligated primers. One would have had a reasonable expectation of success because Favis provides examples of the use of primers with DNA zipcode detection regions (p.563, left col., lns.1-7).

Claim 67 lacks an inventive step under PCT Article 33(3) as being obvious over Belgrader et al (1997) in view of US Patent 5,210,015 to Gefland et al (1993). Belgrader et al teaches the detection of specific nucleic acids using ligatable primers that are amplified and detected via capillary electrophoresis or hybridization to a probe array. Belgrader et al does not teach the analysis of ligation product formation using the cleavage of Q-PCR probes.

Gefland et al teaches the detection of amplified nucleic acids using the nuclease activity of nucleic acid polymerase to release the label portion of a probe (col.13 - Example 1). The cleavage of the probe results in a detectable signal.

It would have been obvious to have modified the method of Belgrader et al to have used the detection probes described by Gefland et al detect the amplified ligation product. One would have been motivated to do so in order to provide an alternative method for detecting ligated primers. One would have had a reasonable expectation of success because Gefland et al provides examples of the detection of amplified nucleic acids using probe cleavage.

Claim 70 lacks an inventive step under PCT Article 33(3) as being obvious over Belgrader et al (1997) in view of Abravaya et al (1995). Belgrader et al teaches the detection of specific nucleic acids pairs of primers that are ligated depending on the sequence of a target nucleic acid. Belgrader et al does not the use of primers directed toward both strands of a double stranded target nucleic acid.

Abravaya et al teaches the detection of mutations using a Gap-ligase chain reaction method (Fig.1; p.676, left column, first full paragraph). The reference describes the use of oligonucleotides directed to both strands (complementary strands) of the

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Supplemental Box
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target nucleic acid (p.675 - Abstract; Fig. 2; Fig. 5A).

It would have been obvious to have modified the method of Belgrader et al to have used the detection probes directed toward both strands of the target nucleic acid as described by Abravaya et al. One would have been motivated to do so in order to increase the reliability of detecting a particular mutation by analyzing both strands of the target nucleic acid.

Claims 1-5 , 28-74 meet the criteria set out in PCT Article 33(4), and thus have industrial applicability because the subject matter claimed can be made or used in industry.